Pflugers Arch - Eur J Physiol (2010) 460:99–108
DOI 10.1007/s00424-010-0799-z

ION CHANNELS, RECEPTORS AND TRANSPORTERS

HL-1 cells express an inwardly rectifying K^+ current activated via muscarinic receptors comparable to that in mouse atrial myocytes

Muriel Nobles · Sonia Sebastian · Andrew Tinker

Received: 19 November 2009 / Revised: 1 February 2010 / Accepted: 3 February 2010 / Published online: 26 February 2010

Abstract An inwardly rectifying K^+ current is present in atrial cardiac myocytes that is activated by acetylcholine (I_{KACh}). Physiologically, activation of the current in the SA node is important in slowing the heart rate with increased parasympathetic tone. It is a paradigm for the direct regulation of signaling effectors by the Gβγ G-protein subunit. Many questions have been addressed in heterologous expression systems with less focus on the behaviour in native myocytes partly because of the technical difficulties in undertaking comparable studies in native cells. In this study, we characterise a potassium current in the atrial-derived cell line HL-1. Using an electrophysiological approach, we compare the characteristics of the potassium current with those in native atrial cells and in a HEK cell line expressing the cloned Kir3.1/3.4 channel. The potassium current recorded in HL-1 is inwardly rectifying and activated by the muscarinic agonist carbachol. Carbachol-activated currents were inhibited by pertussis toxin and tertiapin-Q. The basal current was time-dependently increased when GTP was substituted in the patch-clamp pipette by the non-hydrolysable analogue GTPγS. We compared the kinetics of current modulation in HL-1 with those of freshly isolated atrial mouse cardiomyocytes. The current activation and deactivation kinetics in HL-1 cells are comparable to those measured in atrial cardiomyocytes. Using immunofluorescence, we found GIRK4 at the membrane in HL-1 cells. Real-time RT-PCR confirms the presence of mRNA for the main G-protein subunits, as well as for M2 muscarinic and A1 adenosine receptors. The data suggest HL-1 cells are a good model to study IKAch.

Keywords HL-1 cells · GIRK · Kir 3.1/3.4 · Atrial mouse cardiomyocytes · Muscarinic M2 receptor · Potassium channels · HEK cells

M. Nobles · S. Sebastian · A. Tinker (✉)
Department of Medicine, BHF Laboratories,
University College London, The Rayne Institute,
5 University Street,
London WC1E 6JJ, UK
e-mail: a.tinker@ucl.ac.uk

Introduction

Control of heart rate is a complex process that integrates the function of multiple G-protein-coupled receptors and ion channels. Acetylcholine released from the vagus nerve efferents activates muscarinic receptors on SA nodal cells, initiating a sequence of signaling events. A decrease in heart rate is caused by inhibition of adenylate cyclase via inhibitory heterotrimeric G-proteins and inhibition of If current activity and by the activation of an inwardly rectifying K^+ current [1–4]. Inwardly rectifying potassium channels gated by G-proteins (GIRK) are also present atrial myocytes [5]. The molecular counterparts of these channels have been identified as members of the Kir3.x family of inwardly rectifying K^+ channels and heterotetramers of Kir3.1/3.4 (GIRK1/4) are predominant in the heart [6]. GIRK channels are activated by direct interaction with βγ subunits released from inhibitory heterotrimeric G-proteins upon agonist stimulation of appropriate G-protein-coupled receptors such as the M2 muscarinic and A1 adenosine receptors in the heart. In many ways, this pathway is an exemplar of how G-protein βγ subunits directly regulate a signaling effector [7]. Furthermore, there is emerging evidence that receptor, inhibitory G-protein, regulators of G-protein signaling and channel may be scaffolded together [8–13]. This hypothesis is largely based on the over-expression of components in heterologous expression sys-

tems, and there is an important question as to how applicable such models are when considering signaling in native systems. To some extent, this can be realised by isolating single nodal and atrial myocytes from genetically modified mice or by establishing short-term culture and subsequent viral transfection of acutely isolated cells. However, both these approaches are time-consuming and technically demanding [4, 5, 14–16]. It would be valuable to have a system that reproduced native signaling whilst allowing routine molecular manipulation such as transfection of cDNAs and siRNA duplexes with lipid-based methods.

The HL-1 cell line was derived from AT-1 cardiac myocytes, which are atrial cardiac muscle cells obtained from transgenic mice in which expression of the SV40 large antigen was controlled by the atrial natriuretic factor promoter [17]. HL-1 cells are currently the only cardiomyocyte cell line that divides, spontaneously contracts while maintaining a differentiated cardiac phenotype. The HL-1 cell line has been shown to express functional channels characteristic of cardiac cells: the L- and T-type Ca2+ channels [18], as well as the hyperpolarisation-activated non-selective cation channel I_f [19]. HL-1 cells also beat spontaneously in culture [20]. In this work, we seek to establish if the HL-1 cell line is a potential genetically tractable model for studying the muscarinic regulation of the cardiac G-protein gated inwardly rectifying K^+channel.

Materials and methods

Cell culture

The HL-1 cell line was a gift from Prof. William C. Claycomb (Louisiana State University, Baton Rouge, LA) and was maintained using his recommended protocols. HL-1 cells were cultured under 5% C02 atmosphere in Claycomb medium supplemented with 10% foetal bovine serum, 0.2 mM L-glutamine, 0.1 mM norepinephrine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Sigma). These conditions, including the addition of norepinephrine, were necessary to ensure cell division and maintenance of the cell phenotype. The medium was replaced every day. Cells were grown onto T75 culture flasks pre-coated overnight with 0.03% fibronectine/0.02% gelatine solution. For electrophysiology and imaging experiments, cells were grown on uncoated 13-mm glass coverslips (BDH).

Atrial mouse cardiomyocyte isolation was achieved using a method adapted from a previously published approach [21]. Atrial cells were isolated from adult C57 black mice. All experiments were conducted according to the British Home Office animal welfare guidelines.

At the time of study, mice (equal sex distribution) were 12–14 weeks of age, weighing 20–25 g. Animals were injected 20 min prior to isolation with a bolus of heparin sodium 250 IU. Hearts were rapidly excised from anaesthetized (ketamine 0.01 ml/mg, xylazine 10 mg/ml, atropine 0.06 mg/ml) animals and attached (by the aorta) to a Langendorff perfusion apparatus. Isolated hearts were retrogradely perfused for 10 min with a buffer containing (in millimolar) 113 NaCl, 4.7 KCl, 0.6 KH2PO4, 0.6 Na2HPO4, 1.2 MgSO4.7H2O, 12 NaHCO3, 10 KHCO3, 30 taurine, 10 HEPES, 11 glucose and 10 2,3-butanedione monoxide, followed by a solution containing 1 mg/ml collagenase type V and 1 mg/ml Protease XIV (both from Sigma) and 50 μM calcium. The temperature of the heart and the perfusate were maintained at 35–37°C and saturated with 95% O2–5% CO2. The enzymatic solution was filtered (5 μm) and re-circulated through the heart until the atria were digested (20–35 min), as judged by eye. After perfusion, the atria and appendages were removed, minced and incubated in a fresh solution containing enzymes for an additional 10 min, under shaking condition at 35–37°C and saturated with 95% O2–5% CO2. The tissue pieces were then transferred to 10 ml of fresh (enzyme-free) solution supplemented with 5 mg/ml bovine serum albumin (BSA, Sigma) and 150 μM CaCl2 and, gently triturated with a fire-polished Pasteur pipette for 5–10 min. The resulting suspension was then centrifuged (600 rpm, 5 min); the supernatant was discarded, and the cells were resuspended in fresh BSA-containing solution. This step was repeated twice to completely wash out the enzyme and remove unwanted cellular fragments. Isolated atrial myocytes were resuspended in 2 ml fresh BSA-containing solution, plated on laminin-coated glass coverslips onto 13-mm cell culture coverslips (BDH) and placed in 95% O2–5% CO2 in an incubator at 37°C for 20 min. After 30–45 min, cells were gently resuspended into 2 ml MEM containing foetal bovine serum 10% and penicillin/streptomycin 1%. Cells were used for electrophysiology 1 h after plating for the day.

Cell culture methods and the generation of stable cell lines were as described [22]. HEK293 cells (human embryonic kidney cell line) expressing M2 receptor and GIRK1/4 channel were maintained in minimum essential medium supplemented with 10% foetal calf serum and 727 μg of G418 and 364 μg/ml Zeocin (Invitrogen), at 37°C in humidified 95% O2–5% CO2.

Electrophysiology

Whole-cell patch-clamp current recordings were performed with an Axopatch 200B amplifier (Axon Instruments) using fire-polished pipettes with a resistance of 3–4 MΩ, pulled from filamented borosilicated glass capillaries (Harvard Apparatus, 1.5 mm OD×1.17 mm ID). Data were acquired and analysed by using a Digidata 1322A interface (Axon Instruments) and pCLAMP software (version 10, Axon Instruments). Cells were clamped at −50 mV and bathed in

an extracellular solution containing (millimolar): NaCl 120, KCl 20, CaCl2 2, MgCl2 1, HEPES 10, pH 7.4, while the intracellular solution was (millimolar): K gluconate 110, KCl 20, NaCl 10, MgCl2 1, MgATP 2, EGTA 2 GTP 0.3, pH 7.4. The text and figure legends indicate where modifications were made to this. Drugs were applied by a gravity driven perfusion system. Membrane currents were elicited with a series of 420 ms depolarisation pulses to +60 in 20 mV decrements from a holding potential of −50 mV applied at a frequency of 10 Hz. Current densities were measured at a holding potential of –120 mV, unless otherwise stated.

For the evaluation of current kinetics, a fast perfusion system was used to apply drugs (Rapid Solution Changer, RSC-160, Bio-Logic France). Cells were clamped at −60 mV, the extracellular solution was (millimolar): NaCl 80, KCl 60, CaCl2 2, MgCl2 1, HEPES 10, NaH2PO4 0.33, glucose 10, while the intracellular solution has the same composition as the one mentioned above. After agonist application, current activated with a delay "lag" followed by a rapid rise to peak amplitude "time to peak". After removal of the agonist, the current decays back to baseline. Current activation and deactivation were fitted by a single exponential function $A\exp(-t/\tau)+C$ (where A is the current amplitude at the start of the fit, t is time, τ is the activation or the deactivation time constant, and C is the steady-state asymptote). For each cell, we assessed whether there were any flow artefacts resulting from the pressure of drug application. We did this by applying bath solution from one of the sewer pipes at the beginning of the recordings. Furthermore, to control for variations in positioning of the sewer-pipe system relative to the cell, we calibrated the system by using the kinetics of channel block by 1 mM barium. Block of the current occurred with an initial delay "lag" before reaching equilibrium; it was assumed that this lag reflected the intrinsic delivery time to the cell. The lag time for barium was 194±13 ms (n=16).

Immunofluorescence

Cells were cultured in 13-mm glass-bottom Petri dishes (Mattek), for 48 h. Cells were washed twice with phosphate buffered saline (PBS) containing 0.1 mM CaCl2 and 1 mM MgCl2. Cells were fixed for 20 min, at 4°C, with PBS solution containing 4% paraformaldehyde, and then washed twice with PBS. Then the cells were permeabilised with PBS containing 0.2% Triton and 5% goat serum (Gibco). After 20 min, the solution was removed and replaced by the primary antibody diluted in the above buffer. GIRK4 (APC-027, Alomone Laboratories) and M2 receptor (AMR-002, Alomone Laboratories) antibodies were used at a final dilution of 1/500. Incubation was carried for 90 min, followed by three washes with PBS. Then, the cells were incubated for 60 min with Alexa 488 (goat anti-rabbit A1108, Molecular Probes) in the dark. During incubation with primary and secondary antibodies, 5% goat serum (Gibco) was used to minimise non-specific staining. Appropriate controls (not shown) were undertaken with non-transfected HEK293 cells and incubation of HL-1 and M2 GIRK1/4 cell lines with secondary antibody alone. Cells were then finally washed three times with PBS and imaged with a Biorad confocal microscope. The cells were excited using a 488-nm laser line, and emission was measured using a LP500 filter.

Real-time quantitative RT-PCR

Real-time RT-PCR was performed using Taqman Gene Expression Assays (Applied Biosystems) using either inventoried, made-to-order or custom-made assays. Protocol followed was based on the information provided by the suppliers, which is available on their website http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041280.pdf).

Briefly, hearts were removed from the C57 black wild-type mice, washed with cold PBS and immediately placed in liquid nitrogen. Atrial tissue was ground under liquid nitrogen with a pestle and mortar. We then proceeded with the extraction of RNA using RNeasy kit (cat no. 74104 Qiagen). Similarly, HL-1 cells were harvested from the culture, and RNA was extracted using RNeasy kit (cat no. 74104 Qiagen). cDNA was synthesised using high-capacity cDNA reverse transcription Kit (4368814 Applied Biosystems) from both native cells and HL-1 cell lines. cDNA was quantified, and 50 ng/µl of DNA/20 µl was used for the subsequent real-time expression assay. All genes were assayed in triplicates, and glyceraldehyde-3-phophate dehydrogenase (GAPDH) was used as the house-keeping gene.

Table 1 Kinetics of current activation, desensitisation and deactivation

Cells	Cell capacitance (pF)	I basal pA/pF	I agonist pA/pF	Lag+TTP sec	τac seconds	Percent desensitisation, %	τdeact seconds
HL-1 cell line, n=15	16.4±1	−30±11.6	−81.6±21	3.6±0.5	1.44±0.31	8.4±1.5	6.2±0.5
Mouse atria, n=20	31.5±2.2	−71±6.1[a]	−64±8.5	4.3±0.7	0.63±12	3.2±0.9[a]	4±1
M2 GIRK1/4 cell line, n=11	22.2±1.8	−44±4.3	−125±49	7.4±0.9	1.61±0.21	6±1.3	8.5±0.9

[a] $p<0.05$ compared with HL-1 cell line. Data are shown as mean±SEM

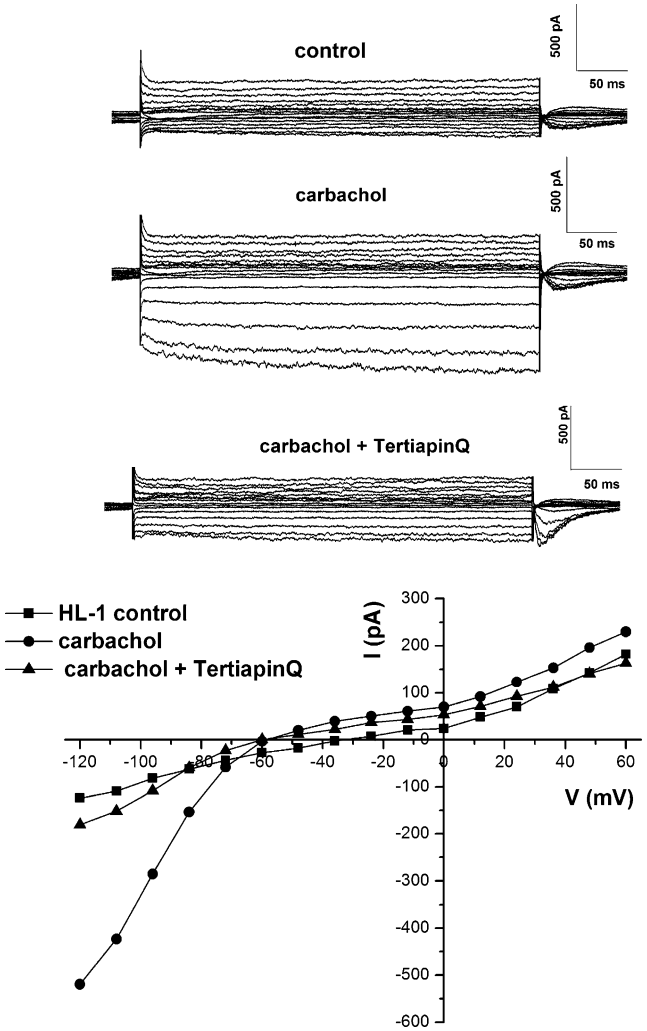

Fig. 1 K^+ current activated by carbachol in HL-1 cells. Carbachol activates an inwardly rectifying K^+ current in HL-1 cells. Sample voltage-clamp recordings and current voltage relationships are shown

Statistical analysis

Data are presented as mean ± SEM unless otherwise indicated. A Student's *t* test was used for statistical comparison and $*p<0.05$ and $**p<0.01$.

Results

Carbachol-induced potassium current in the HL-1 cell line

Using the whole-cell configuration of the patch-clamp technique, we studied receptor-mediated GIRK currents in HL-1 cells and compared this with a HEK293 stable line expressing the Kir3.1\3.4 channel complex along with the M2 receptor as a positive control (denoted M2 GIRK1/4 cell line). In HL-1 cells and the M2 GIRK1/4 cell line, there was a basal inwardly rectifying current that reversed at approximately the equilibrium potential for K^+ in these solutions (Table 1 and Figs. 1 and 2). The application of the non-specific muscarinic receptor agonist carbachol (10 μM) further increased the inward currents (Table 1). The current elicited by carbachol was blocked by the GIRK channel inhibitor tertiapin-Q (100 mM, Figs. 1, 2 and 3a, $p<0.01$). Pertussis toxin catalyses the ADP ribosylation of the Gi/o α subunit at a cysteine residue four amino acids from the C-terminal end of the protein, and the modified G-proteins are unable to participate in signaling. Treatment of HL-1 cells with pertussis toxin (100 ng/ml for 16 h overnight) led to an inhibition of the carbachol-induced current in HL-1 cells (Fig. 3a, $p<0.01$).

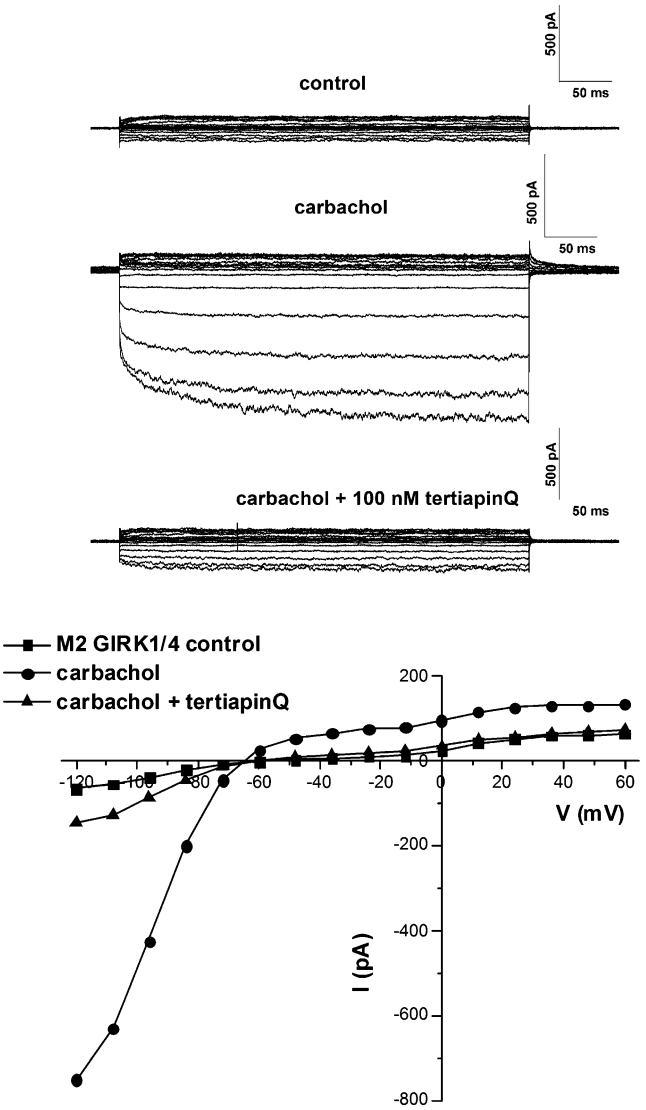

Fig. 2 K^+ current activated by carbachol in HEK293 stable cell line. Carbachol activates an inwardly rectifying K^+ current in a stable HEK293 cell line expressing the M2 receptor and the GIRK1 and GIRK4 channel subunits. Sample voltage-clamp recordings and current voltage relationships are shown

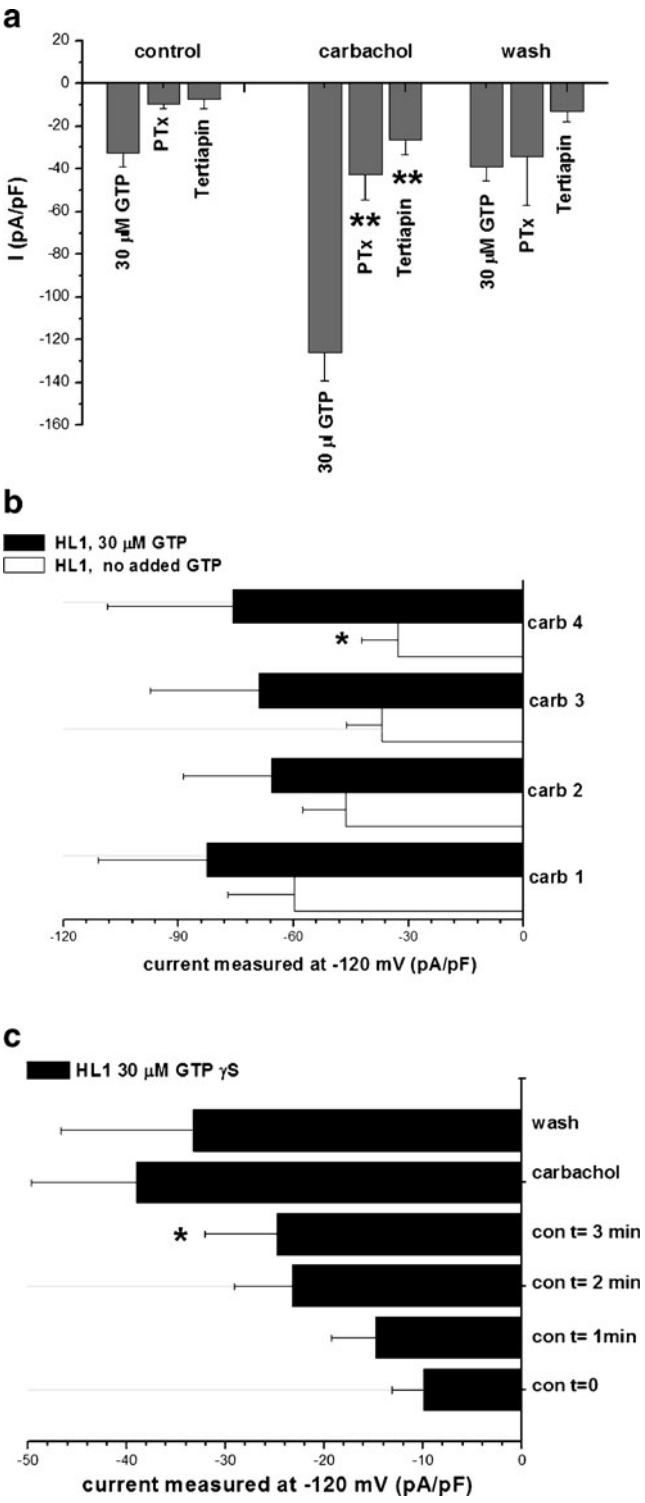

Fig. 3 Dependence of K^+ current activation on G-proteins in HL-1 cells. **a** The bar charts summarise the results obtained. Treatment with pertussis toxin (100 ng/ml for 16 h) or tertiapin-Q (100 nM) led to inhibition of the carbachol-activated current ($p<0.01$ for both). **b** Carbachol (10 μM) was applied four times for 1 min, with 1-min interval between applications, in the presence or absence of 0.3 mM GTP. Decline of the current elicited by carbachol was obtained only in the set of experiments where GTP was omitted ($p<0.05$). **c** Currents were recorded for 3 min after break-in before addition of 10 μM carbachol in the presence of 30 μM intracellular GTPγS. Currents activated with dialysis of GTPγS into the cell ($p<0.05$ compared with current at $t=0$) and carbachol evoked smaller currents

These data support the potential presence of a G-protein-gated inwardly rectifying K^+ current analogous to IKACh in HL-1 cells. To further characterise this current, we looked at the GTP-dependency of the channel activation by using different concentrations and varying the GTP species in the intracellular solution in the patch pipette. The results are shown in Fig. 3b, c. Four sequential applications of carbachol every minute in the presence of 30 μM GTP in the pipette solution led to a stable agonist-activated current on each application of the agonist (Fig. 3b). However, when GTP was omitted, we observed a progressive decline in the agonist-induced current. When compared, the currents at the fourth application of carbachol was significantly ($p<0.05$) lower when GTP was omitted from the patch pipette.

Thus, the magnitude of the receptor-activated current is dependent on GTP. In another set of experiments, we used the non-hydrolysable GTP analogue, GTPγS (30 μM), in the intracellular solution (Fig. 3c). The current was recorded every minute for a 3-min period after break-in, and we observed a progressive increase of basal inwardly rectifying currents ($p<0.05$ compared with the basal current at the beginning of the recordings). Further application of carbachol led to a small increase in the current. We conclude that the current in the HL-1 cell line can be activated by using the non-hydrolysable form of GTP, GTPγS.

We have found that one of the more sensitive methods of measuring changes in signaling "strength" is to quantify the dynamics of current modulation after the application of a saturating dose of agonist. Carbachol was applied to cells using a fast application system for 20 s and then rapidly removed. We compared the kinetics of current activation, the degree of rapid desensitisation at the end of agonist application, and the kinetics of deactivation of the HL-1 cells with those of freshly isolated mouse atrial cardiomyocytes. Currents activated with a lag and then an exponential rise before reaching a peak amplitude (lag+time to peak or "TTP"). A single exponential function was used to fit the latter activation phase (τac). With prolonged agonist application, current amplitude wanes as the response desensitises (acute desensitisation), and upon removal of agonist, it deactivates back to baseline levels. Deactivation of currents following agonist removal could also be well-fitted by a single exponential function (τdeact). The kinetics of activation and deactivation of HL-1 resemble those of the mouse atrial cells (Fig. 4 and Table 1), the only significant difference being a smaller percentage of acute desensitisation of the GIRK current in the mouse atria compared with the HL-1 cell line and increased basal currents ($p<0.05$ both).

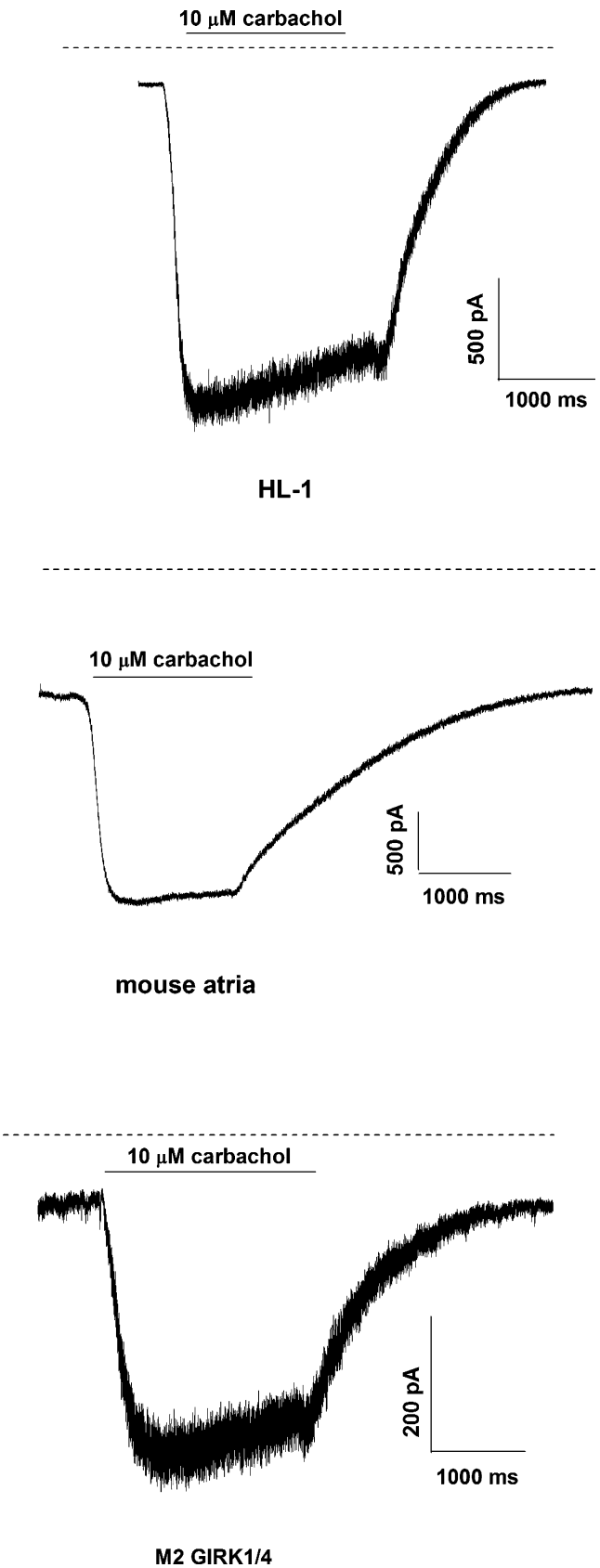

Fig. 4 Kinetics of activation of the K^+ current. Representative current traces recorded from HL-1 cells, native mouse atrial myocytes and the M2 GIRK1/4 cell line. Cells were clamped at −80 mV and 10 μM carbachol was applied for 20 s. The mean parameters are summarised in the Table. *Dotted lines* indicate zero current

Immunofluorescence confirms the presence of GIRK4 and M2 in the HL-1 cell line

We used an antibody against the GIRK4 subunit to look at the presence and distribution of the channel in the HL-1 cell line. All the cells show staining (Fig. 5). As a positive control, we stained the M2 GIRK1/4 cell line with the same GIRK4 antibody. In an analogous manner, the M2 receptor was also present in the HL-1 cells (Fig. 5). The pattern of staining for GIRK4 in the HL-1 cells appears to be different from that in the HEK293 stable line. Thus, we performed two additional controls. Firstly, in HEK293 cells without GIRK4 expression, the GIRK4 antibody does not stain the cells (Fig. 5). Secondly, incubation of the M2 GIRK1/4 cell line with only the secondary antibody, and not the primary, does not lead to staining (Fig. 5).

Real-time quantitative RT-PCR

We also examined the expression of G-proteins ($G_{i\backslash o}$ and G_s), the M2 muscarinic, A1 adenosine receptor and the GIRK1 and GIRK4 channel subunits at the transcriptional level using real-time RT-PCR. Data are expressed as ΔCt compared with the "house-keeping" gene GAPDH (a higher value reflects lower expression and single cycle difference reflects a twofold expression difference assuming high-efficiency PCR). There are two notable features with the data. The first is that the general pattern of expression is similar between the two lines. For example, in the inhibitory family of G-proteins Gi2 and Go α subunits are the most highly expressed compared with Gi1 and Gi3. The only exception to this might be the ratio of GIRK1/4 expression. The second point is that the relative expression compared with the house-keeping gene of all the proteins is lower in the HL-1 cells compared with the native atrial cells (Fig. 6).

This is discussed further below.

Discussion

GIRK channels are activated through various seven helical receptors coupled to inhibitory heterotrimeric G-proteins (Gi/Go). Receptor activation after agonist binding leads to GDP release, GTP binding on the Gα subunit, and dissociation of the heterotrimeric G-protein subunits, Gα-GTP and Gβγ. The βγ subunits then interact with the GIRK channel, causing an increase in the open probability of the channel complex. The classical model for this

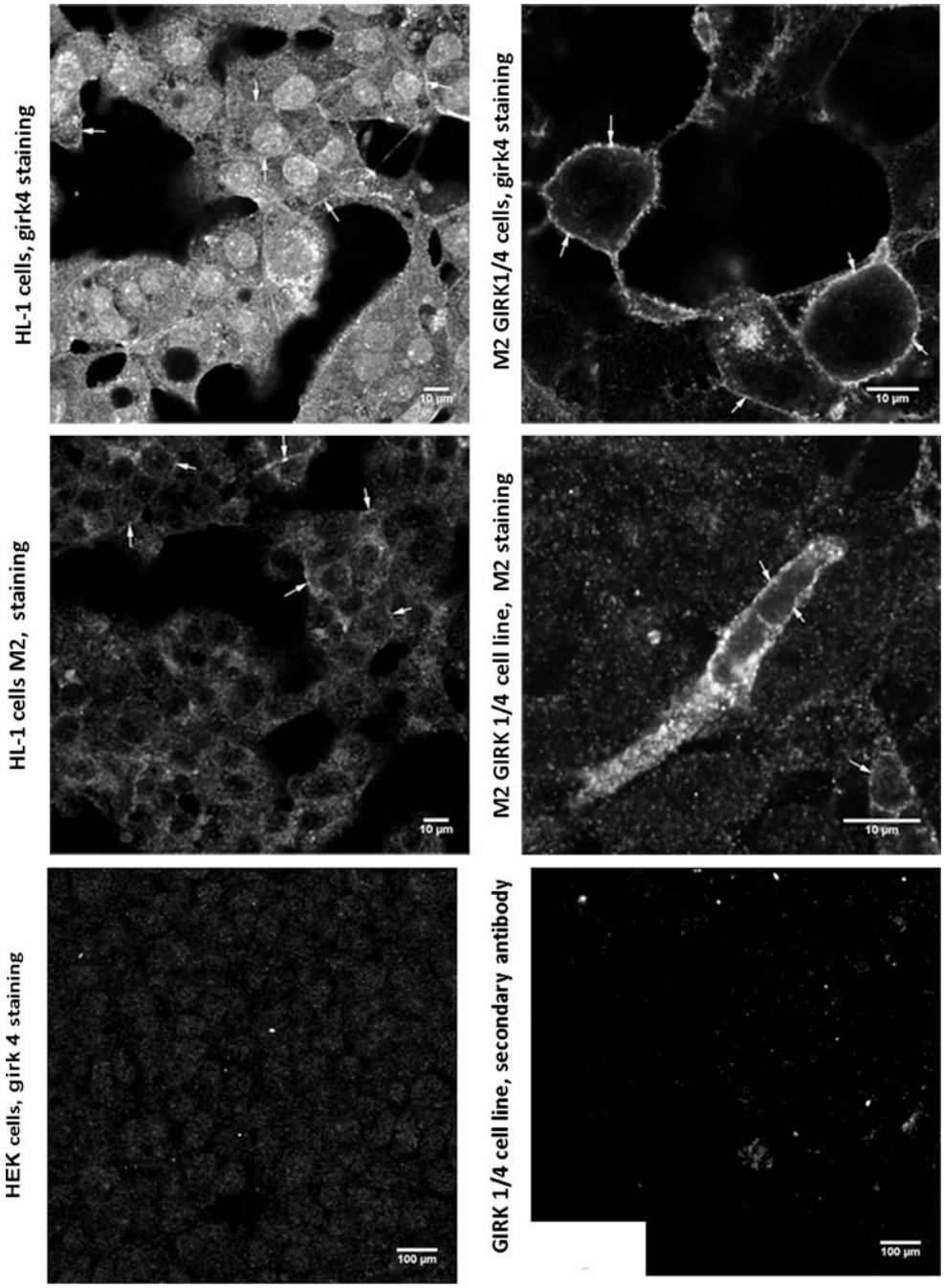

Fig. 5 Immunostaining of HL-1 cells. Immunofluorescent staining of HL-1 cells and the M2 GIRK1/4 cell line stained for GIRK4 and the M2 receptor, respectively. *Arrows* show potential membrane localisation. In the *lower panels* of the figure, two controls are shown. In parental HEK293 cells without GIRK4 expression, the GIRK4 antibody does not stain the cells. Incubation of the M2 GIRK1/4 cell line with only the secondary antibody and not the primary does not lead to staining

cascade includes free diffusion of the membrane components and interaction by random collision [23]. However, this model has been challenged, and the emerging view is that components of the signaling cascade form a multimeric complex at the cell membrane. There is published work showing that inhibitory Gα subunits directly associate with the channel and control channel gating [9–11, 22, 24] and that G-protein-coupled receptors and regulators of G-protein signaling may participate in this multimeric complex [8, 13, 25, 26]. Proteomic studies indicate other signaling components may be present too [27]. The development of these models has been heavily dependent upon the overexpression of signaling components in heterologous expression systems. There are questions about whether such schemes are applicable to the signaling system in native atrial or nodal cardiac myocytes and also about which specific isoforms of the various proteins are important. For example, it seems that RGS4 and Gi2 are critical in nodal cells [15, 28]. However, these experiments used mice with global genetic deletion of the proteins, and this is time-consuming, expensive and not always feasible to undertake. It would be valuable to have a system that endogenously expressed the signal transduction cascade and was readily manipulated using standard transfection

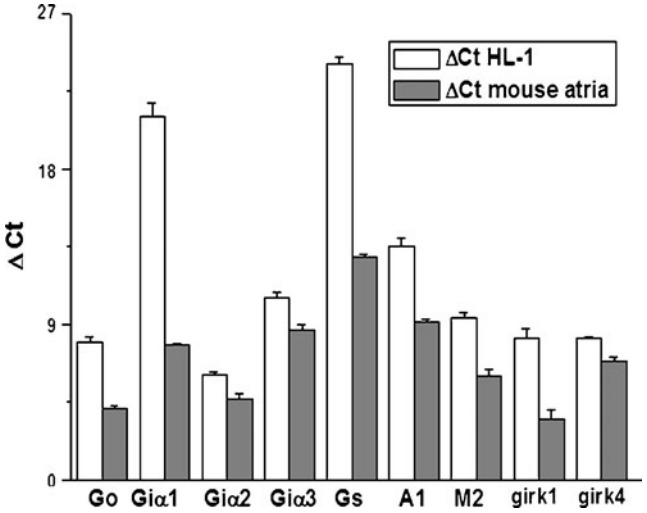

Fig. 6 Quantitative real-time RT-PCR. ΔCt values in quantitative real-time RT-PCR experiments performed as indicated in the methods. Data are shown as mean ± SD (n=3 for HL-1 cells and n=6 from two mice)

techniques. There are also ethical considerations for reducing the use of animals for such work. The aim of this work was to investigate whether the HL-1 cell line could fulfil such a role.

In HL-1 cells, we isolated an inwardly rectifying K^+ current that was activated by a muscarinic agonist and inhibited by the relatively specific blocker tertiapin-Q [29]. The receptor modulation was inhibited by pertussis toxin and dependent on the provision of GTP, characteristics of the G-protein gated Kir3.0 family of channel as opposed to the constitutively active Kir2.0 group also present in cardiac cells. We examined whether GIRK4 (Kir3.4) was present using immunofluorescence, and indeed, it was. GIRK4 is a characteristic component of the cardiac current as opposed to the molecular constituents of the current in central neurones and neuroendocrine cells [6, 30]. In order to characterise further the GIRK current recorded in the HL-1 cell line, we looked at the kinetics of activation and deactivation using a fast perfusion protocol. The kinetics, current densities and degree of acute desensitisation were either not significantly different from those in acutely isolated mouse atrial cells or were only marginally different. We have found the signaling dynamics to be a good indicator of the status of the transduction cascade, and the assay can report perturbations and differences with high sensitivity [8, 31, 32]. The fact that the kinetics are so closely matched is good evidence that the pathway is highly comparable. Immunofluorescence revealed the presence of GIRK4 in HL-1 cells; however, there was significant localization in an intracellular pool. The physiological significance of this is as yet unclear; however, it is noteworthy that cell signaling can influence the trafficking of ion channels to and from the plasma membrane [33].

We also investigated the relative expression of G-protein alpha subunits, the A1 and M2 G-protein-coupled receptors and GIRK1 and GIRK4 using real-time quantitative RT-PCR. The rank order of expression was similar in the two cell types except for GIRK1 and GIRK4 (discussed below). For example, both Gi2 and Go were the best expressed of the inhibitory heterotrimeric G-proteins. This is consistent with our recent in vivo studies in genetically modified mice [28] in which we saw phenotypes in heart rate regulation in Gi2 and Go global knockout mice but none in a combined knockout of Gi1 and Gi3. A second issue is that, relative to a control gene, the expression of the assayed proteins is reduced in HL-1 cells. Glyceraldehyde-3-phosphate dehydrogenase is a cytoplasmic glycolytic enzyme, whereas the proteins whose relative transcriptional expression we assayed are extrinsic or intrinsic membrane proteins. We have noticed that native atrial myocytes are larger than the HL-1 cells whose size is quite heterogenous (see Table 1 for cell capacitances). For a cytoplasmic enzyme, concentration will be proportional to the cell radius to the 3rd power ($r3$) whilst density in the plasma membrane will be scaled to $r2$. Thus, as cell size increases, it might be expected that all else being equal you might need relatively more mRNA for a cytoplasmic enzyme than for one that encodes a membrane protein. Whilst this general issue of how cells adapt gene expression to changing cell size has not been studied, to our knowledge, it is clear that failure to adapt in cardiac hypertrophy may underlie some of the apparent electrical remodeling [34].

There were some differences between native atrial cells and the HL-1 cells. Firstly, in native atrial myocytes, there were increased basal current densities. This could accounted for by an increase in free $G\beta\gamma$ due to differences in endogenous basal signaling or the expression of activators of G-protein signaling or guanine nucleotide dissociation inhibitors [35, 36]. Alternatively, there may be higher levels of expression of the Kir2.0 family of inward rectifiers in the atrial cells, but the systematic exploration of this possibility would require extensive further work and is a topic for future investigation. In general, in atrial cells, there is only modest relative expression of classical strong inward rectifiers generated by Kir2.0 compared with the G-protein-gated channel [37]. A further possibility is that there may be differences in the preponderance of GIRK4 homomultimers. Our real-time RT-PCR studies revealed that the expression of GIRK1 and GIRK4 in HL-1 cells was approximately equivalent, whilst in atrial cells, GIRK4 expression was reduced compared with GIRK1. The increase in currents upon muscarinic receptor activation were however similar. Secondly, we found that rapid desensitisation was less prominent in the atrial cells. In our studies to date, on acutely isolated mouse (C57 black mice specifically) atrial cells, we have found this phenom-

enon to be quite variable—prominent in some cells, but not present in others. We have previously argued that it reflects the dynamics of the G-protein cycle [38], and presumably, there may be some subtle differences between atrial cells and HL-1 cells and also within atrial cells as a population.

The existence of the IKACh current in HL-1 cells may have additional uses. There is accumulating evidence that there is constitutive activity of GIRK currents in atrial fibrillation [39, 40]. In addition, these currents are largely expressed in supraventricular tissues, and thus, Kir3.1/3.4 represents a potential drug target for treating atrial fibrillation [41]. Furthermore, rapid pacing of HL-1 cells leads to transcriptional remodeling characteristic of atrial fibrillation [42]. Thus, HL-1 cells might be a useful model for adaptive electrophysiological changes that occur in atrial fibrillation. In summary, we show that HL-1 cells possess a G-protein-gated inwardly rectifying K^+ current that is analogous in properties and receptor-mediated regulation to its counterpart in native atrial myocytes.

Acknowledgments This work was supported by the Wellcome Trust. We are grateful to Prof. William Claycomb for providing the HL-1 cell line.

References

1. Baruscotti M, Robinson RB (2007) Electrophysiology and pacemaker function of the developing sinoatrial node. Am J Physiol Heart Circ Physiol 293:H2613–H2623
2. Noma A, Trautwein W (1978) Relaxation of the ACh-induced potassium current in the rabbit sinoatrial node cell. Pflügers Archiv 377:193–200
3. Robinson RB, Siegelbaum SA (2003) Hyperpolarization-activated cation currents: from molecules to physiological function. Annu Rev Physiol 65:453–480
4. Wickman K, Nemec J, Gendler SJ, Clapham DE (1998) Abnormal heart rate regulation in GIRK4 knockout mice. Neuron 20:103–114
5. Wellner-Kienitz MC, Bender K, Meyer T, Bunemann M, Pott L (2000) Overexpressed A(1) adenosine receptors reduce activation of acetylcholine-sensitive K(+) current by native muscarinic M(2) receptors in rat atrial myocytes. Circ Res 86:643–648
6. Krapivinsky G, Gordon EA, Wickman K, Velimirovic B, Krapivinsky L, Clapham DE (1995) The G-protein-gated atrial K^+ channel IKACh is a heteromultimer of two inwardly rectifying $K^{(+)}$-channel proteins. Nature 374:135–141
7. Ford CE, Skiba NP, Bae H, Daaka Y, Reuveny E, Shekter LR, Rosal R, Weng G, Yang C-S, Iyengar R, Miller RJ, Jan LY, Lefkowitz RJ, Hamm HE (1998) Molecular basis for interactions of G protein bg subunits with effectors. Science 280:1271–1274
8. Benians A, Nobles M, Hosny S, Tinker A (2005) Regulators of G-protein signalling form a quaternary complex with the agonist, receptor and G-protein: a novel explanation for the acceleration of signalling activation kinetics. J Biol Chem 280:13383–13394
9. Clancy SM, Fowler CE, Finley M, Suen KF, Arrabit C, Berton F, Kosaza T, Casey PJ, Slesinger PA (2005) Pertussis-toxin-sensitive Galpha subunits selectively bind to C-terminal domain of neuronal GIRK channels: evidence for a heterotrimeric G-protein-channel complex. Mol Cell Neurosci 28:375–389
10. Nobles M, Benians A, Tinker A (2005) Heterotrimeric G-proteins precouple with G-protein coupled receptors in living cells. Proc Natl Acad Sci USA 102:18706–18711
11. Peleg S, Varon D, Ivanina T, Dessauer CW, Dascal N (2002) G (alpha)(i) controls the gating of the G protein-activated K(+) channel, GIRK. Neuron 33:87–99
12. Riven I, Iwanir S, Reuveny E (2006) GIRK channel activation involves a local rearrangement of a preformed G protein channel complex. Neuron 51:561–573
13. Zhang Q, Pacheco MA, Doupnik CA (2002) Gating properties of GIRK channels activated by Galpha(o)- and Galpha(i)-coupled muscarinic m2 receptors in Xenopus oocytes: the role of receptor precoupling in RGS modulation. J Physiol 545:355–373
14. Bechem M, Pott L, Rennebaum H (1983) Atrial muscle cells from hearts of adult guinea-pigs in culture: a new preparation for cardiac cellular electrophysiology. Eur J Cell Biol 31:366–369
15. Cifelli C, Rose RA, Zhang H, Voigtlaender-Bolz J, Bolz SS, Backx PH, Heximer SP (2008) RGS4 regulates parasympathetic signaling and heart rate control in the sinoatrial node. Circ Res 103:527–535
16. Gehrmann J, Meister M, Maguire CT, Martins DC, Hammer PE, Neer EJ, Berul CI, Mende U (2002) Impaired parasympathetic heart rate control in mice with a reduction of functional G protein betagamma-subunits. Am J Physiol Heart Circ Physiol 282:H445–H456
17. White SM, Constantin PE, Claycomb WC (2004) Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. Am J Physiol Heart Circ Physiol 286:H823–H829
18. Xia M, Salata JJ, Figueroa DJ, Lawlor AM, Liang HA, Liu Y, Connolly TM (2004) Functional expression of L- and T-type Ca2+ channels in murine HL-1 cells. J Mol Cell Cardiol 36:111–119
19. Sartiani L, Bochet P, Cerbai E, Mugelli A, Fischmeister R (2002) Functional expression of the hyperpolarization-activated, non-selective cation current I(f) in immortalized HL-1 cardiomyocytes. J Physiol 545:81–92
20. Claycomb WC, Lanson NA Jr, Stallworth BS, Egeland DB, Delcarpio JB, Bahinski A, Izzo NJ Jr (1998) HL-1 cells: a cardiac muscle cell line that contracts and retains phenotypic characteristics of the adult cardiomyocyte. Proc Natl Acad Sci USA 95:2979–2984
21. Boyle WA, Nerbonne JM (1992) Two functionally distinct 4-aminopyridine- sensitive outward K^+ currents in rat atrial myocytes. J Gen Physiol 100:1041–1067
22. Leaney JL, Milligan G, Tinker A (2000) The G protein a subunit has a key role in determining the specificity of coupling to, but not the activation of G protein-gated inwardly rectifying K^+ vchannels. J Biol Chem 275:921–929
23. Tolkovsky AM, Levitzki A (1978) Mode of coupling between the beta-adrenergic receptor and adenylate cyclase in turkey erythrocytes. Biochemistry 17:3795
24. Huang CL, Slesinger PA, Casey PJ, Jan YN, Jan LY (1995) Evidence that direct binding of G beta gamma to the GIRK1 G protein-gated inwardly rectifying K^+ channel is important for channel activation. Neuron 15:1133–1143
25. Fujita S, Inanobe A, Chachin M, Aizawa Y, Kurachi Y (2000) A regulator of G protein signalling (RGS) protein confers agonist-dependent relaxation gating to a G protein-gated K^+ channel. J Physiol 526(Pt 2):341–347
26. Lavine N, Ethier N, Oak JN, Pei L, Liu F, Trieu P, Rebois RV, Bouvier M, Hebert TE, Van Tol HH (2002) G protein-coupled receptors form stable complexes with inwardly rectifying potassium channels and adenylyl cyclase. J Biol Chem 277:46010–46019

27. Nikolov EN, Ivanova-Nikolova TT (2004) Coordination of membrane excitability through a GIRK1 signaling complex in the atria. J Biol Chem 279:23630–23636
28. Zuberi Z, Birnbaumer L, Tinker A (2008) The role of inhibitory heterotrimeric G-proteins in the control of in-vivo heart rate dynamics. Am J Physiol Regul Integr Comp Physiol 295:R1822–R1830
29. Drici MD, Diochot S, Terrenoire C, Romey G, Lazdunski M (2000) The bee venom peptide tertiapin underlines the role of I (KACh) in acetylcholine-induced atrioventricular blocks. Br J Pharmacol 131:569–577
30. Yamada M, Inanobe A, Kurachi Y (1998) G protein regulation of potassium ion channels. Pharmacol Rev 50:723–757
31. Benians A, Leaney JL, Milligan G, Tinker A (2003) The dynamics of formation and action of the ternary complex revealed in living cells using a G-protein-gated K^+ channel as a biosensor. J Biol Chem 278:10851–10858
32. Benians A, Leaney JL, Tinker A (2003) Agonist unbinding from receptor dictates the nature of deactivation kinetics of G-protein gated K^+ channels. Proc Natl Acad Sci USA 100:6239–6244
33. Hu K, Huang CS, Jan YN, Jan LY (2003) ATP-sensitive potassium channel traffic regulation by adenosine and protein kinase C. Neuron 38:417–432
34. Marionneau C, Brunet S, Flagg TP, Pilgram TK, Demolombe S, Nerbonne JM (2008) Distinct cellular and molecular mechanisms underlie functional remodeling of repolarizing K^+ currents with left ventricular hypertrophy. Circ Res 102:1406–1415
35. Blumer JB, Smrcka AV, Lanier SM (2007) Mechanistic pathways and biological roles for receptor-independent activators of G-protein signaling. Pharmacol Ther 113:488–506
36. Wiser O, Qian X, Ehlers M, Ja WW, Roberts RW, Reuveny E, Jan YN, Jan LY (2006) Modulation of basal and receptor-induced GIRK potassium channel activity and neuronal excitability by the mammalian PINS homolog LGN. Neuron 50:561–573
37. Dobrev D, Graf E, Wettwer E, Himmel HM, Hala O, Doerfel C, Christ T, Schuler S, Ravens U (2001) Molecular basis of down-regulation of G-protein-coupled inward rectifying K(+) current (I(K, ACh) in chronic human atrial fibrillation: decrease in GIRK4 mRNA correlates with reduced I(K, ACh) and muscarinic receptor-mediated shortening of action potentials. Circulation 104:2551–2557
38. Leaney JL, Benians A, Brown S, Nobles M, Kelly D, Tinker A (2004) The rapid desensitisation of G-protein gated inwardly rectifying K^+ currents is determined by the G-protein cycle. Am J Physiol Cell Physiol 287:182–191
39. Dobrev D, Friedrich A, Voigt N, Jost N, Wettwer E, Christ T, Knaut M, Ravens U (2005) The G protein-gated potassium current I(K, ACh) is constitutively active in patients with chronic atrial fibrillation. Circulation 112:3697–3706
40. Voigt N, Maguy A, Yeh YH, Qi X, Ravens U, Dobrev D, Nattel S (2008) Changes in I K, ACh single-channel activity with atrial tachycardia remodelling in canine atrial cardiomyocytes. Cardiovasc Res 77:35–43
41. Savelieva I, Camm J (2008) Anti-arrhythmic drug therapy for atrial fibrillation: current anti-arrhythmic drugs, investigational agents, and innovative approaches. Europace 10:647–665
42. Mace LC, Yermalitskaya LV, Yi Y, Yang Z, Morgan AM, Murray KT (2009) Transcriptional remodeling of rapidly stimulated HL-1 atrial myocytes exhibits concordance with human atrial fibrillation. J Mol Cell Cardiol 47:485–492